Spring 5-4-2020

Rapid Antibiotic Susceptibility Testing Platform for Direct Clinical Samples

Terrance Zhang
terrance.zhang@uconn.edu

Rapid Antibiotic Susceptibility Testing Platform for Direct Clinical Samples

Author: Terrance Zhang

Project Supervisors: Joerg Graf Ph.D, Guoan Zheng Ph.D

Honors Advisor: Joerg Graf Ph.D

Keywords: Multidrug Resistance, Galvo Speckle-Scanning, Microfluidic Cell-Culture Device

TABLE OF CONTENTS

Table of Figures

Table of Tables

Abstract

Infectious diseases and septicemia are two of the major causes of death in the U.S., necessitating rapid treatment of septic patients with proper, efficacious antibiotics. Unfortunately, the emergence and spread of multidrug-resistant bacteria are continuously being aggravated by an abuse in antibiotic prescription at a clinical and agricultural level. It is known that antibiotic resistance evolves through the sequential accumulation of multiple mutations in bacteria, which is accentuated by prolonged exposure of bacteria to ineffective antibiotics when implementing traditional septicemia treatment. The goal of this project is to develop a novel, easy-to-use AST platform for rapid antimicrobial susceptibility profiling to reduce the incidence and mortality rates, and to reduce inappropriate antibiotic usage commonly associated with invasive pathogens. As time progresses, antibiotic resistance becomes more prevalent and so does the need to effectively test antibiotics on any given bacteria strain in a timely manner. This project will utilize an *E. coli* bacteria and antibiotic plating method combined with a single-cell-level lensless imaging system to achieve this task. Here we propose a device that incorporates the working principle of a speckle-scanning pictographic lensless imaging scheme to visualize (in real-time) the growth of single cells of bacteria in a microscopic population under different antibiotic types and different concentrations of respective antibiotics. This system may further be optimized by directly imaging a blood sample, effectively bypassing the time it takes to perform traditional blood sampling, culture isolation, and culture propagation for antibiotic susceptibility testing. Our model organism will be *Escherichia coli* K-12 - a gram-negative facultative anaerobe with a generation time of 20-30 minutes.

Introduction

Antibiotic resistance is a major healthcare concern worldwide [1]. Antibiotics are over-prescribed and misused, which significantly impacts the effectiveness of the drug. With every hour of delay until a patient is introduced to an antibiotic, their survival rate decreases by 7.6% [2]. Antibiotic susceptibility testing takes time that a patient suffering from infection doesn't have. Typically a general antibiotic is administered rather than testing for the most effective strain, which in many cases can lead to the bacterial microorganisms building a resistance to the drug meant to remove them.

Antibiotic resistance took around 700,000 people's lives in 2014 and is projected to grow greatly [3]. The economic toll associated with increased time spent in hospitals is also expected

to grow from $35 billion to nearly $100 trillion by 2050[4]. Rapid antibiotic susceptibility testing will mitigate these effects as the correct antibiotic will be able to be chosen at the start of patient care.

The gold standard for antibiotic susceptibility testing takes 10-36 hours to correctly identify the best antibiotic to prescribe [5]. In the meantime, patients are typically given a wide array of antibiotics while they await lab results. We intend to lower the chances of antibiotic resistance by removing the need to prescribe ineffective antibiotics while awaiting lab results. We will do this by reducing lab times from 12-36 hours to 1-2 hours. We will develop a platform to test various types of antibiotics at multiple concentrations and their effect on the proliferation of certain bacteria. Using a lensless imaging platform we will be able to determine the most effective type and concentration of antibiotic at a much faster rate.

The proposed project starts with culturing bacterial samples on agar plates and propagating the cultures. Six concentrations of up to four different antibiotics will be introduced to separate chambers (for a total of 24 chambers). Alternatively, the testing platform may consist of two channels - one for water and one for a concentrated antibiotic solution. The channels will be connected to a middle agar channel, allowing for the establishment of a concentration gradient of a single antibiotic (this unit is to be repeated four times for four types of antibiotics). The growth of bacteria in response to the antibiotic will be observed using a high-resolution lensless imaging technology that will allow for the observation of single-cell activity, and a comparison of instances of growth will be used to determine the minimum inhibitory concentration of the sample.

Literature Review

In 1928, scientist Alexander Fleming was crowned with the discovery of a miracle drug - Penicillin [6]. This accidental antibiotic discovery has gone on to save millions of lives by effectively treating diseases and conditions that were once considered life-threatening and fatal. The versatility of this drug has led to non-specific administration and abuse, ultimately leading to the accelerated development of antibiotic-resistant bacterial pathogens. The rise of multidrug-resistant pathogenic lifeforms has since threatened global health, as propagation of antibiotic resistance via horizontal transfer (from bacteria-to-bacteria) of plasmids containing drug-resistant genes is rapid and often uncontested [6]. In modern-day hospital settings, patients who show signs of septicemia (acute blood infection) are promptly treated with a “cocktail” of broad-range antibiotics in hopes of inhibiting further damage to the host. Four hours after the delivery of the initial antibiotic(s), blood samples are collected to be tested for the presence and identification of microorganisms. Upon positive identification, further extensive tests are required to identify the antibiotic susceptibility profile of each individual strain present. This common

protocol can take anywhere from 24 hours to several days, depending on the growth rate and efficacy of preliminary broad-range antibiotics. This lag time and abuse of broad-range antibiotics are speculated to increase the chances of the pathogenic strains developing antibiotic resistance, as bacterial communities grow accustomed to their static environment. Thus, every hour of delay (or every hour of improper treatment) is estimated to decrease the survival rate of sepsis by approximately 7.6% [2]. The development of Rapid Antibiotic Susceptibility Testing systems that incorporate accurate identification with determination of the minimum inhibitory concentration (MIC) is of high priority. Such a system allows for simultaneous identification of the pathogen whilst determining efficacious treatment options in a fraction of the time required for traditional analysis protocols to significantly decrease the mortality rate of septicemia [5].

Proper treatment of diseased patients and inhibition of the development of antibiotic resistance requires scientists and clinicians to establish the susceptibility profile of pathogenic bacteria before treatment with broad-range antibiotics. The rapid identification of a bacterial susceptibility profile enables accurate treatment with an efficacious antibiotic that prevents the possibility of bacterial propagation, development of resistance, and horizontal transfer of plasmids [7]. Under traditional treatment protocols, infectious bacteria are exposed to a constant drug concentration that partially or completely kills or inhibits the growth of the base strain. This method, while capable of inhibiting the progress of septic shock, imposes a selective advantage for drug-resistant mutants under gradual rates of evolution [8]. Phenotypic Antibiotic Susceptibility Testing (ASTs) are based on the detection of bacterial growth in the presence of different types of antibiotics, different concentrations of respective antibiotics, and growth on either a liquid or solid media. Modern-day AST standards (such as broth microdilution) achieve reliable result readouts in 12-36 hours. However, this significant time delay fails to guide treatment for the early, critical stages of infection. By the time that a patient is symptomatic, the pathogen will have already inflicted significant damage on the patient and elicited a major immune response - not to mention the patient will also begin to display a significant risk to secondary contacts [7]. The issue arises from the lack of current research. Studies have suggested mutational trajectories that may result in mutations that enable antibiotic resistance, but single point mutations are often insufficient for the development of a high level of antibiotic resistance. Thus, the development of a Rapid Antibiotic Susceptibility Testing system is beneficial for a clinical setting because it severely restricts the amount of time a bacterial strain is exposed to constant levels of antibiotics, subsequently limiting the potential for mutation. By rapidly introducing an efficacious antibiotic to a diseased patient, scientists and clinicians may

kill all susceptible strains and dispel the survival of spontaneously occurring mutants [8]. Our Rapid AST solution rapidly determines growth and inhibitory conditions of organisms with optimal growth conditions. By integrating multiple exposure sites onto a single plate, the new proposition may test several antibiotics and different concentrations of respective antibiotics at once. To further reduce analysis time, we will incorporate the working principle of a speckle-scanning pictographic lensless imaging scheme to visualize (in real-time) the growth of single cells of bacteria in a small population. By limiting the population size to a microscopic scale and comparing relative rates of cell division, we hope to capture every instance of cell growth in a limited time frame. These instances may then be compared to other exposure sites to compare relative growth rates and determine optimal inhibitory conditions. An ultimate reduction of diagnostic time would further incorporate direct blood testing to skip over preliminary blood sampling and culturing while monitoring for bactericidal effects as opposed to bacteriostatic effects, as the former mechanism ensures elimination of the pathogenic agent whilst the latter mechanism prevents growth and does not prevent conjugation.

Currently, there are many methods used for AST including automated and manual systems. The AST methods that are presently practiced, “in the clinical microbiology labs are accurate, but are either labor-intensive or time-consuming, leading to long wait times to obtain AST results” [5, 9, 13, 14]. The problem with these methods is that they all require a lengthy amount of time to provide results. Current AST manual systems include but are not limited to; the Broth microdilution method, the agar dilution method, the disk diffusion method, and Etest. The Current AST automated systems are VITEK, Sensititre, and BD phoenix. Even though both the manual and automated systems for rapid AST use bacterial growth or metabolism to determine the effect of antibiotics, automated systems still take the same time as manual systems to obtain antibiotic susceptibility profiles, “since they require pure bacterial isolates and use optical methods for interpretation of results” [5, 10, 12, 14, 18]. Today, many clinical laboratories have switched from manual systems to automated systems as they are easier to use, don’t require any labor, and have a more effective workflow. Both manual and automated systems have their advantages and disadvantages, and their common disadvantage is time. Two qualities of rapid AST are accuracy and time, with accuracy being efficient, the amount of time to produce results is not. This is due to current technologies require either pure cultural isolates or use observation of changes to bacterial metabolism. The main focus of emerging and future methods is to reduce the time as much as possible by having the same working accuracy for results. Researchers are now going after rapid AST systems that can bypass the need for pure isolate, use patient samples directly, and improve the sample processing time. With the

abilities of these characteristics, the future of patient treatment can be attained, the use of broad antibiotics can be reduced, and AST outcomes can prosper.

Table 1: Summary of the state of antibiotic susceptibility testing platforms [5].

System	**Approach**	**Time for AST**
Broth Microdilution (Golden Standard)	Media containing different antibiotics tested against the pathogen of interest	24-36 hours
Agar Dilution	Antibiotic incorporated directly into agar plates, bacteria inoculated on the surface. Multiple plates may be concocted sequentially and incubated simultaneously.	16-24 hours (per plate)
Disk Diffusion	Antibiotic-impregnated filter discs placed on agar surface pre-inoculated with pathogen	16-36 hours
Etest	Plastic strip impregnated with gradually decreasing concentrations of antibiotics placed on agar surface pre-inoculated with pathogen	24-36 hours
VITEK System	Measures light attenuation by optical scanners for growth/no growth detection in micro-wells with different antibiotics	12-36 hours
Sensititre	Fluorescence technology used to monitor the activity of enzymes produced by test organism emitting fluorescence	12-24 hours
BacterioScan FLLS	Uses laser light source with scattered intensity measurements for accurate Optical Density readings in the presence of antibiotics	10-24 hours
BD Pheonix Automated ID and AST	Automated broth microdilution that uses a redox indicator to detect growth, capable of reading 99 panels at once	4-16 hours
Micro-scan Walk Away	Colorimetric readings based on photosensors for optical detection of bacteria in 40-96 panels	4.5-7 hours (up to 18 hours)
MALDI-TOF Mass Spectrometry	Culture inoculated into different suspensions containing antibiotics, and growth rate is calculated from spectra	3-5 hours

Agonistic Real-Time PCR	Isolates propagated on agar or broth with antibiotics, and PCR run to determine inhibition of growth	2-4 hours
Our System	**Ptychographic lensless imaging over a large field of view. Determines growth or no growth at single-cell resolution level of multiple panels at once**	**30min-2 hours**

Broth Dilution Methods

Current broth macro- and microdilution methods utilize multiple panels (wells), each filled with a range of antibiotics at different concentrations. These methods require that the bacteria of interest be isolated, grown on agar plates, and suspended in media. An equal amount of the inoculated media is introduced into all wells except for the positive and negative controls. Following an incubation period of 6-24 hours (or more), the wells are observed for signs of growth. This application may be extended to agar plate applications. In this case, antibiotics are directly incorporated into respective liquid agar samples, which are then poured into respective plates. After the agar has set, bacteria is directly inoculated onto the surface and the plate is incubated and checked for growth [5, 13].

Diffusion Methods

Antibiotic susceptibility testing methods that utilize diffusion of antibiotics include Disk Diffusion and Etests. In both of these tests, a bacterial sample isolated from the patient is directly swabbed onto a sterile agar plate. The Disk Diffusion method utilizes a series of predetermined concentrations of antibiotic-laden discs (of filter paper). These antibiotic-laden discs are placed onto the surface of the agar, and the plate is allowed to incubate for up to 24 hours. Following this, the diameter of the zones of inhibition around the discs are recorded to determine the efficacy of the tested antibiotics against the bacterial sample [5, 11, 13].

The Etest method utilizes the diffusion mechanism of the Disk Diffusion method. In contrast, Etests utilize impermeable plastic strips impregnated with a gradient of a given antibiotic (from high to low concentration). These strips are placed onto the inoculated agar, and the plate is incubated for up to 24 hours. The growth patterns may then be used to determine the minimal inhibitory concentration of an antibiotic, determined as the minimum concentration on the Etest at which bacterial growth is inhibited [5].

Automated Methods

The VITEK System, Sentititre Plate, BacterioScan FLLS, BD Pheonix Automated ID and AST, Micro-Scan Walk-Away, and MALDI-TOF systems are all based on the Broth Microdilution. These systems utilize scanners to measure light attenuation, fluorescence, indicators, or mass spectrometry to determine optical density as a factor of growth rate. Given the sensitivity of scanners over human vision, these systems advertise significantly decreased times required to compile a bacterial sample's antibiotic susceptibility profile [5, 9, 10, 11, 12, 13, 14, 15, 16].

However, scanning systems rely on reasonably large changes in the existing bacterial population to determine the antibiotic susceptibility profile. While they address issues associated with incubation and imaging, they do not address delays associated with pre-AST stages (clinical sampling, bacterial isolation, etc.). We are seeking to design a low-cost, rapid antibiotic susceptibility testing platform that enables active imaging of individual bacterial cells under a wide field-of-view [7, 19]. By monitoring singular instances of fission in bacterial cells subjected to select antibiotics under a concentration gradient, we hope to drastically reduce the time of antibiotic susceptibility profiling down to the generation time of a given bacterial species. Furthermore, we hope to extrapolate this design and utilize raw clinical blood samples to further decrease the time required for AST. Given the complexity and cost associated with automated systems, it was determined that these methods would be outside the realm and budget of this design.

Materials and Methods

Needs Analysis & Function Specifications

The final goal of this design is to successfully integrate with a single-cell-level lensless imaging system that is capable of tracking bacterial fission in a large population of cells (ideally >50). We are creating a platform to appropriately test the effect of various concentrations of antibiotics on bacteria. This culture will then be imaged by a predetermined device, therefore compatibility is key. Biosafety level is ranked the lowest. It was made clear due to lab resources that a Biosafety Level 1 is ideal (with up to Biosafety Level 2 available), but this does not hold more weight than the other specified needs. Finally, for this device to be applicable to the rapid AST market, it must be portable for ease of use in medical settings. A portable model assumes an efficient and ergonomic design, and the capacity to utilize multiple platforms in rapid succession.

Table 2: Functions (Specifications)

Function	Specification
Allow for viewing access	Use of clear PDMS for platform and agar growth medium on top of the device for straight forward imaging
Test for multiple antibiotics	Use of 2-3 different types of antibiotics
Allows for a linear concentration gradient	Two regions connected by a channel allow for linear diffusion of antibiotics into an aqueous buffer
Allows for direct access	Ability to add antibiotic and draw samples at any time while experimenting
Allows for material interaction	Allow bacteria and antibiotic to interact
Propagates growth of bacteria	Agar channel allows for surface growth and adhesion of E.coli
Prevents adhesion	Prevent adhesion of the antibiotics and bacteria to the surface of the device
Can be easily manufactured	Low cost of the 3D printed mold, high quantity of PDMS available, and relatively high curing rate of PDMS allows for many devices to be fabricated

Concept

The task at hand was to create a petri-dish-like assembly that could allow for a comparison of how various concentrations of different types of antibiotics affect bacterial growth. The main issue we faced was the need to identify which concentration of antibiotic was affecting specific areas of the bacteria culture as a means to determine the minimum inhibitory concentration of an antibiotic on a particular strain of bacteria (in this case, *E. coli*). It was decided that the need for different concentrations throughout the culture can be solved by establishing a concentration gradient via linear diffusion of antibiotics into an aqueous buffer. This eliminates the need for complete sectioning of the culture and a clustered multi-well configuration. The meeting concluded with the decision to diffuse water and antibiotics from two wells placed on opposite ends of the bacterial culture (agar) and base the applied

concentrations off distance from the source (via linear diffusion approximation). The hope is that the antibiotic concentration is the strongest when it is closest to the antibiotic well and linearly dilutes into a negligible concentration near the well filled with a buffer solution. The design is still in the early stages and it is unclear how exact concentrations will be calculated after diffusion. The design created is shown in Figures 1-2.

The initial design considered was to print molds of a microfluidic box. Using the below diagrams (Figures 1-2), Part A was to be placed on top of Part B, and a PDMS solution poured through the holes in the top. After proper gelling and curing of the solution, a nutrient agar solution inoculated with bacteria would be poured through the top, and the resulting product would be a device that could accurately demonstrate bacteria growth and susceptibility to various antibiotics. As agar media is porous, bacteria would be capable of evenly growing over the surface of the agar platform. However, a few issues arose with this initial design. Primarily, Part A contains a hole in the center, which was initially designed to allow for removal of the part after solidification of both PDMS and agar. However, this would cause a large number of issues. The hole is not large enough to encompass all of the agar, so a significant amount would be displaced during this process. Additionally, the sides of the center wall, when removed, would cause leakage between the two wells. Finally, Part B doesn't allow easy removal of the entire system - it would be hard to separate the PDMS base from the mold base. Therefore, the next design needed three main improvements.

1. Easier removal of Part A from agar
2. No gap between agar and PDMS
3. Easier removal of PDMS from Part B

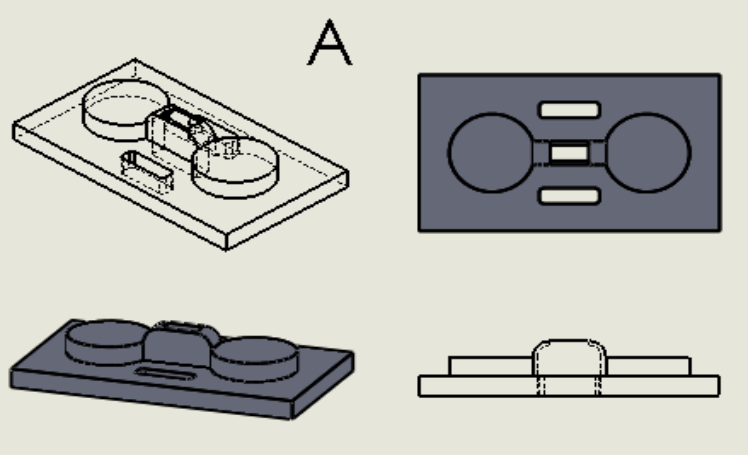

Figure 1: Conceptual Design of Microfluidic Box Top - PDMS

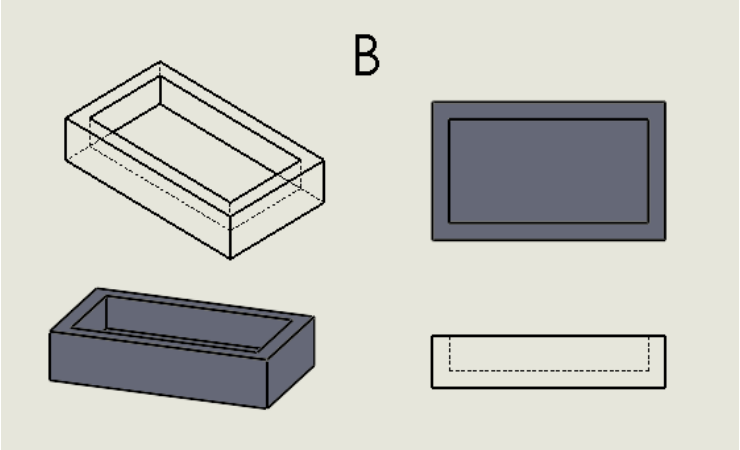

Figure 2: Conceptual Design of Microfluidic Box Base

Preliminary Design

It was originally intended that the device be composed of several microfluidic channels that integrated the same antibiotic-buffer concentration gradient. However, this design was ruled out as the method in which bacteria were to be imaged required for the formulation of fluid channels capable of housing single bacterium cells. This would lead to difficulty due to the macroscopic resolution of any available printing device, the varied morphology of bacterial cells, and the difficulty imagining growth rate in small populations. At any given time, a small subset of bacterial cells are in the process of active binary fission (replication), and the incidence of bacterial division may be increased by increasing our sample size (the bacterial population) from a single cell to several colony-forming units (CFUs). The issues that were found in the initial box design were resolved by remodeling. The new design is pictured in Figures 3-5. Improvement 1 needed an easier removal of Part A from the PDMS. This is addressed by removing the hole from the center of Part A, and the addition of Part C. Now, Part A will only be used for the gelling of PDMS. Once the PDMS has solidified, Part A will be removed and Part C will take its place. The agar solution will be poured through the center of Part C and removed once the agar solidifies. This system also remediates Improvement 2 - there will no longer be a gap between the agar and PDMS due to the thin mold wall. The final improvement in this design is the removal of the bottom of Part B. The product can be made on a flat surface such as in a Petri dish. After solidification, the product can be removed by sliding a knife around the interior walls of the box base.

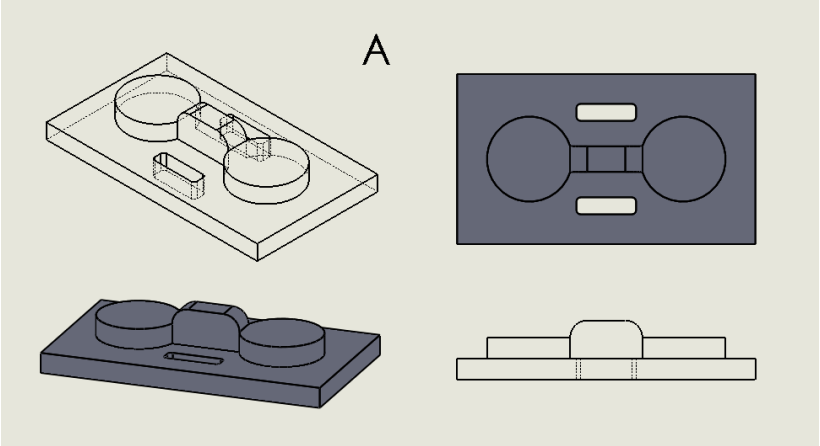

Figure 3: Initial Design of Microfluidic Box Top - PDMS

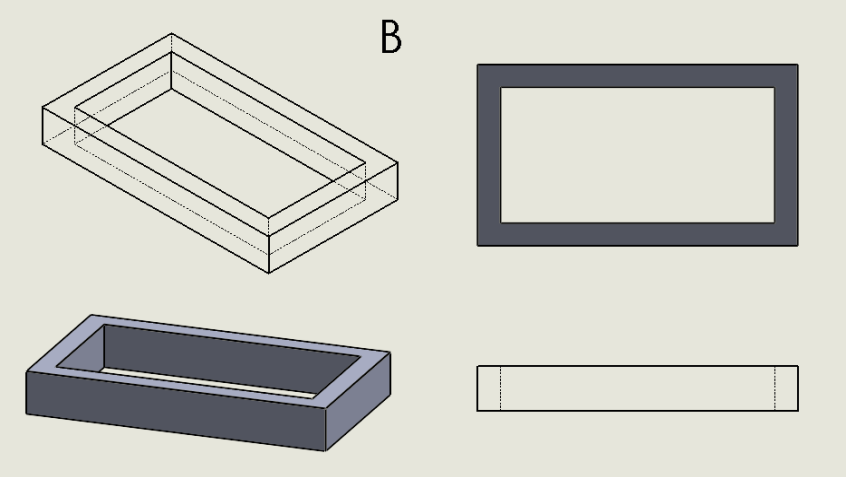

Figure 4: Initial Design of Microfluidic Box Base

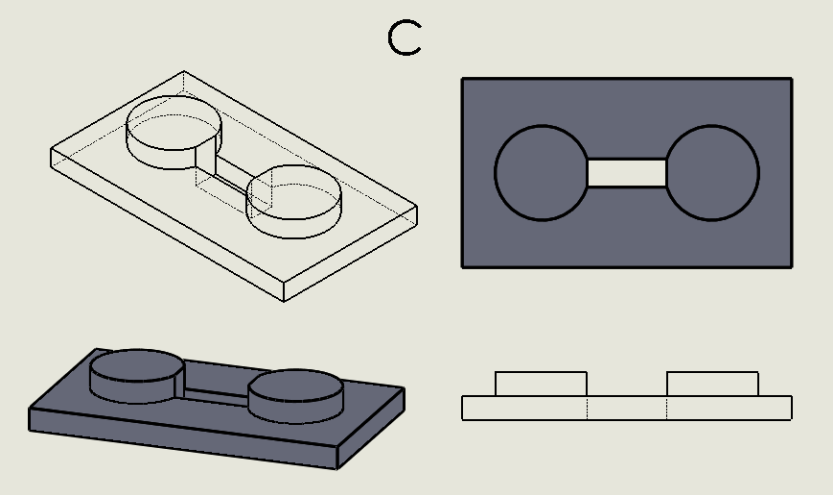

Figure 5: Initial Design of Microfluidic Box Top - Agar

A growth-oriented alternative design utilized direct blood samples from patients to create the agar platform on which bacteria are to be cultured and tested for antibiotic susceptibility. The theory behind this design utilizes the concept of 5-10% mammalian blood-enriched agar plates, currently used in microbiology laboratory settings as a means to cultivate bacteria that are both simple and fastidious, as well as differentiate the bacterial colonies based on hemolytic properties. It is known that blood cultures isolated from patients take approximately 12 to 72 hours to yield positive results for identification and antibiotic susceptibility testing protocols [5, 9, 10, 11, 13, 17]. It is theorized that by directly introducing patient blood samples to a broth-agar media (e.g. melted tryptic soy agar) and pouring a very thin layer of the agar onto the microfluidic device, there would be little to no need for preliminary incubation of the bacterial samples. The bacteria initially present in the blood will now be present within the solidifying thin-agar platform and is free to grow on the surface. As the amount of agar to be used in each microfluidic well is minimal, one blood sample may be used to test multiple antibiotics and multiple concentrations of respective antibiotics. We consulted Professor Joerg Graf, Professor and Associate Department Head for Graduate Research and Education in Molecular and Cell Biology, who told us that the design was entirely feasible for septic patients (albeit the best method would utilize metabolic dyes, which would likely be outside of the realm of our budget). By directly introducing patient blood samples to the microfluidic antibiotic susceptibility testing device, we hope to eliminate preliminary blood testing and ultimately reduce the antibiotic testing time so that it is equal to the generation time of common bacterial species (e.g. 20-30 minutes for *E. coli*).

However, a few issues arose with this alternative design. According to Professor Joerg Graf, bacteria (especially fastidious strains) grow slower in the presence of blood than they would normally grow in the presence of standard nutrient broths/agars due to the presence of cytokines and immune cells. Additionally, the lensless pictographic imaging system that will be utilized, while capable of detecting color, will have great difficulty differentiating bacterial cells from blood culture cells (erythrocytes, platelets, lymphocytes, etc.) due to the small size of the bacterial cells (0.2-2.0μm) in relation to erythrocytes (4-10μm) and lymphocytes (7-20μm). It is possible that an overabundance of erythrocytes and lymphocytes in the agar will prevent an accurate assessment of bacterial concentrations and bacterial growth, even if the imaging system is capable of capturing cross-sectional images. Ultimately, this alternative design is limited by the number of bacterial cells present in the blood sample of a patient who is septic. At the onset of clinical symptoms of sepsis, the concentration of bacteria in a blood sample is very

low (1-300 CFU/mL), so it is highly recommended that the blood sample undergo preliminary subculturing and biochemical identification before antibiotic susceptibility testing protocols [5].

Alternative Designs

The primary component in the creation of the rapid antibiotic susceptibility test is the platform on which the bacterial sample would be cultured and exposed to antibiotics. When the rapid assessment of antibiotic susceptibility profiles was factored into the design, it was determined that the conceptual anaerobic incubation conditions were not optimal for the goal of this project. Additionally, the conceptual design could not be moved during the testing process to avoid disequilibrium of the diffusion gradient and bacterial population. To integrate our device with a higher-level imaging system, the antibiotic effectiveness would need to be modeled on a microfluidic assembly that is minimally obstructive and can be moved freely during any stage of testing.

Thus, a new preliminary design was modeled off of the popular “Etest” antibiotic susceptibility testing system. The new AST device consisted of three independent wells connected with a porous strip that would establish the antibiotic gradient. This connecting strip enables the diffusion and the establishment of an antibiotic gradient over the middle culturing (agar) well by inserting itself into both end wells (antibiotic solution and distilled water, respectively). As the strip would possess a significantly smaller cross-sectional area than the conceptual channel-design, the preliminary device would be able to reduce the impact that kinetic disequilibria would have on the diffusion gradient by minimizing the flow of liquids.

The box design is pictured in Figures 6 and 7. This box will be used as a mold for a Polydimethylsiloxane (PDMS) solution. Part A will only be used for the gelling of PDMS. Once the PDMS has solidified, Part A will be removed and Part C taking its place. The agar solution will be poured through the center of Part C and removed once the agar solidifies. After solidification of PDMS, the product can be removed by sliding a knife around the interior walls of the box base.

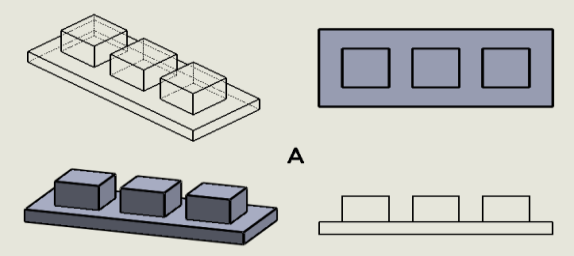

Figure 6: Preliminary Design of Microfluidic Box Top

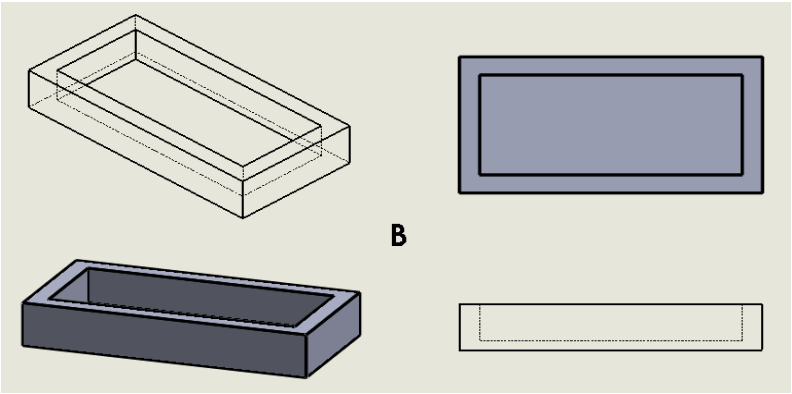

Figure 7: Preliminary Design of Microfluidic Box Base

Preliminary prototyping using the 3D printers and resin printer determined that the minimum resolution that may be obtained will not be small enough to house single bacterial cells (typically 0.2-0.4um). As a result of this, a microfluidic channel that visualizes the growth of small populations (<5) of bacterial cells should not be utilized, and we opted for a design that favored significant bacterial populations (ideally >50 cells). It was also determined that a metabolic fluorescence compound or a live-dead strain of active bacterial cells would be the absolute fastest way to determine an antibiotic's minimum inhibitory concentration, as we would simply test for active enzymatic activity (5-10 minutes) as opposed to testing for binary fission in bacteria (30min - 8 hours). While the provided lensless pictographic imaging system is capable of detecting colors, this method was later found to cost several hundred, if not thousands, of dollars to complete a fluorescence analysis.

It was determined that under improper storage conditions (greater than -4℃), bacterial colony samples were metabolically active and were unable to survive for greater than 3 weeks without re-isolation and subsequent incubation in fresh nutrient medium. As a result of this feasibility study, it was determined that the *E. coli* samples are to be stored in an ultra-low or cryogenic freezer at conditions of -40 degrees centigrade or less.

Bacterial populations inoculated into a liquid nutrient-agar broth at a temperature of 37 degrees centigrade were capable of growing on the surface and within the nutrient-agar medium once solidified. This enables the inoculation of patient bacterial samples into a liquid medium and subsequent introduction and cultivation of bacterial samples directly on the microfluidic device via the pouring method, as previously mentioned.

Feasibility Study

Testing of the conceptual (initial) design of the rapid AST prototype with blue dye determined that kinetic disruption of the device during any phase of testing would result in disequilibrium of the passive diffusion gradient and ruin the establishment of the antibiotic concentration gradient (Figures 8 and 9). The generation time of our *E. coli* bacterial sample was extended from approximately 25 minutes in controlled aerobic conditions to almost 60 minutes under anaerobic conditions. Thus, the initial design was not utilized as it was poorly efficacious when considering the goal of rapid AST

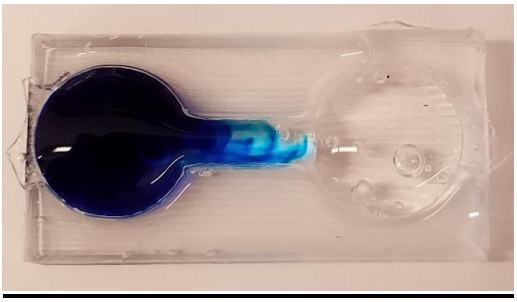

Figure 8. Methylene blue dye (light) and distilled water (right well)

Figure 9. Methylene blue dye (light) and distilled water (right well) concentration gradient after slight disturbance

The preliminary design offered greater flexibility than the initial design, as the porous strip was able to establish an antibiotic concentration gradient under aerobic growth conditions. As suspected, the poor resolution of the 3D printer combined with the inverted-mold design introduced a significant number of microscopic air bubbles into the design. Attempts to remove these bubbles using low-pressure vacuums failed and the resulting devices were both fragile and non-functional after one use, as the resultant air pockets penetrated the cured wells (Figure 10).

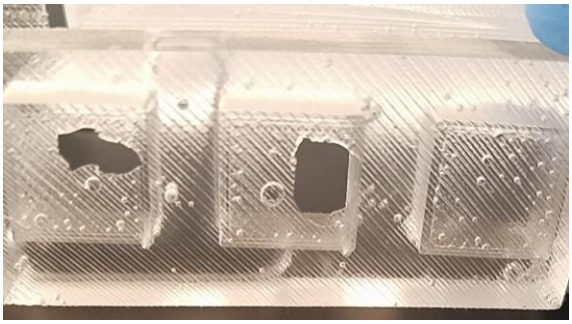

Figure 10: Damage of the microfluidic device due to air bubbles

Data and Results

Final Microfluidic Design

To successfully create the mold for the microfluidic box testing platform, the designs created through SolidWorks must be printed using a 3D printer. There were a few iterations of this printing due to the need for a mold that was conducive to PDMS solidification. The resolution of printing and the size of the print were altered for optimal results. Preliminary prototyping using the 3D printers and resin printer determined that the minimum resolution that may be obtained will not be small enough to house single bacterial cells, 0.2-0.4um. For initial testing purposes, the mold was printed in a larger size then the final product would be created in.

It was thought that the combination of utilizing an inverted-mold design and printing the device using a 3D printer would introduce microscopic air pockets that would create cavities during the curing process. As a result, the preliminary design was further optimized and simplified to eliminate the need to introduce an inverted mold into the uncured PDMS, as seen in our final optimized mold design (Figure 11).

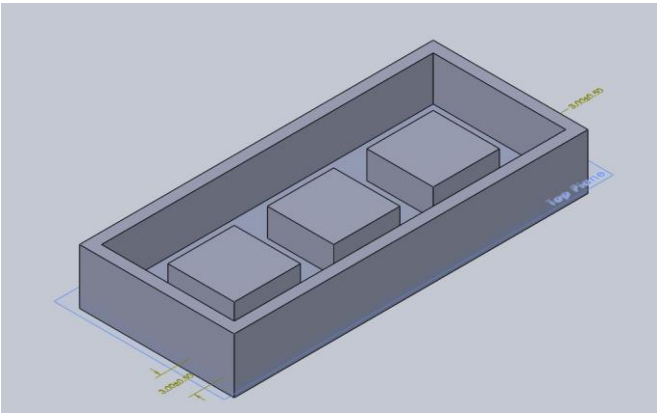

Figure 11: Final Optimized Design of Microfluidic Box Mold

The 3-D printed box mold was then used to create the petri-dish like testing platform. The PDMS was assembled by mixing Sylgard 184 silicone elastomer base and Sylguard 184 silicone elastomer curing agent in a 10:1 ratio in an open container. The open container containing the mixture was placed under a vacuum for one hour to remove excess air bubbles. After no air bubbles remain visible, the mixture was poured into the 3D print of the microfluidic box, and the mold (with the uncured PDMS) was placed into the vacuum for an additional hour. Afterward, the device was allowed to cure in a 65ºC oven for 6-8 hours. The device was then removed from the oven, and the PDMS device was removed from the printed mold using tweezers.

This new optimized design successfully created a prototype of the rapid AST device with minimal presence of air bubbles. While it was more difficult to remove this device from the mold, it was determined that the overall integrity, transparency, and efficacy of the final optimized design far surpassed the conceptual/initial and preliminary designs.

Figure 12. Final optimized mold design (top) and rapid AST device made from solidified PDMS (bottom)

Preliminary Testing the Prototype:

To simulate an E-test, Luria broth agar was first melted and held at 45 degrees centigrade to keep the agar in its liquid state. An inoculating loop was used to introduce a small population of *E. coli* into a sterile nutrient broth, and the inoculated broth was vortexed to evenly distribute bacterial cells. 3 mL of the inoculated broth was poured into 10 mL of the liquid agar, and the resulting inoculated liquid agar was gently vortexed. The liquid agar was rapidly poured into a sterile petri dish and allowed to set at room temperature. A strip of sterile filter paper was placed in the middle of the set agar. 4μL of distilled water and 4μL of $5x10^{-3}$ μg/μL ampicillin solution

were introduced to respective ends of the strip simultaneously. The simulated E-test was incubated at 37ºC incubator at atmospheric conditions for 18 hours. Figure 13 shows the simulated E-test, and Figure 14 shows the zone of inhibition at the minimal inhibitory concentration under a light microscope.

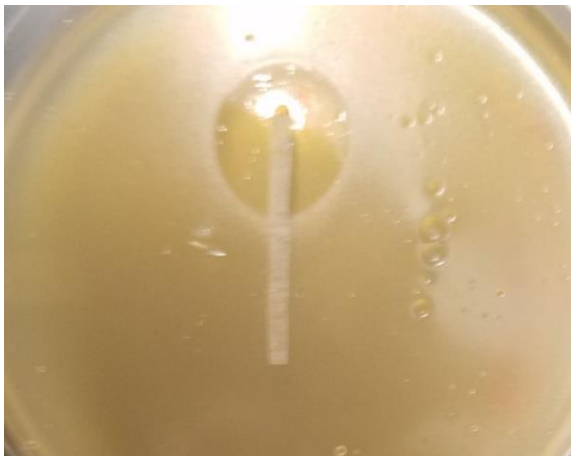

Figure 13: Simulated E-Test using filter paper

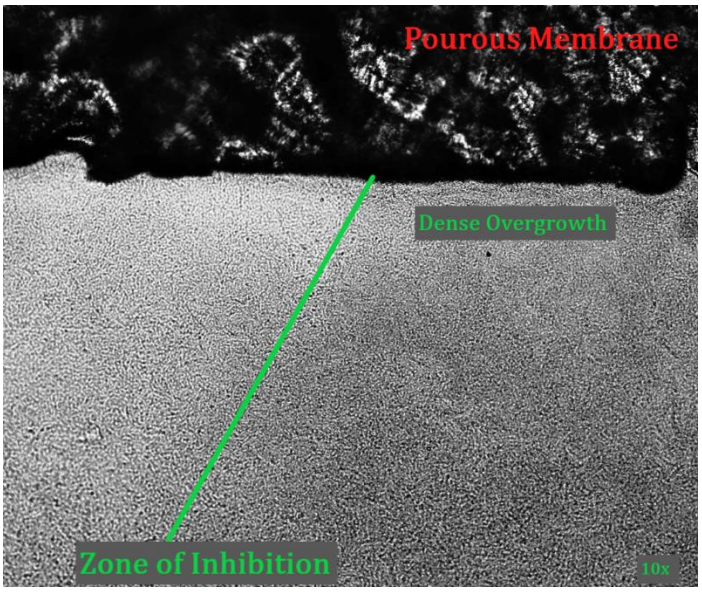

Figure 14: Zone of Inhibition at the minimal inhibitory concentration of ampicillin using simulated E-test (image taken through light microscopy)

Preliminary Results

Using the device, we filled the left and right wells with $5x10^{-3}$ µg/µL ampicillin solution and distilled water, respectively. Sterile Luria broth agar was melted and held at 45 degrees

centigrade to prevent solidification. A sterile nutrient broth solution was inoculated with a colony of *E. coli*, and the inoculated broth was vortexed. 3mL of the inoculated broth was poured into 10mL of the liquid agar, and the resultant inoculated agar was gently vortexed to evenly distribute bacteria. An initial snapshot of the solidified agar well was taken at 0 minutes, as seen in Figure 15 (4x magnification) and Figure 16 (10x magnification). The inoculated agar was then rapidly introduced to the middle well of the device prototype.

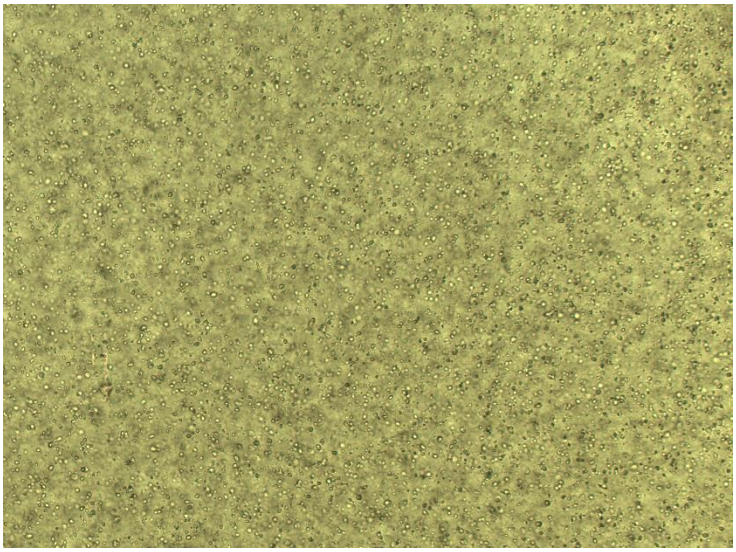

Figure 15: Initial snapshot image of the solid inoculated agar taken under light microscopy at 4x magnification

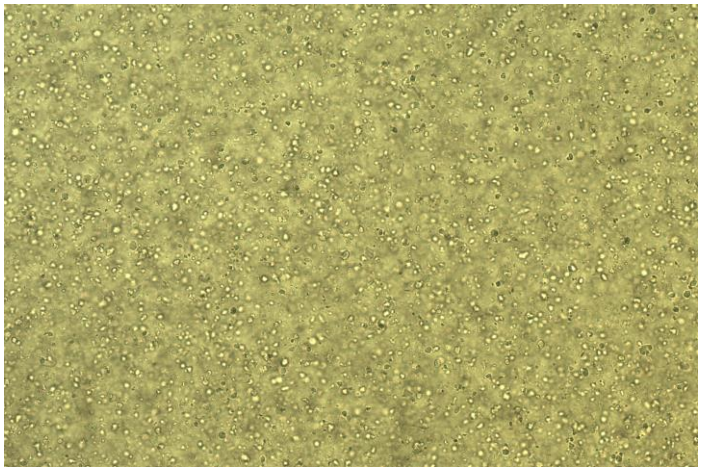

Figure 16: Initial snapshot image of the solid inoculated agar taken under light microscopy at 10x magnification

A thin strip of filter paper was placed over one-fourth of the middle well's agar width, and the ends of the filter paper were introduced to the liquid wells simultaneously. The device was incubated in atmospheric conditions at 37ºC for 8 hours, and a final snapshot of the growth was documented. The zone of inhibition may be seen in Figure 17 (4x magnification) and Figure 18 (10x magnification). Figure 19 is an image of the zone of inhibition under 20x magnification. At this magnification, the field-of-view is not wide enough to determine the boundary between growth and inhibition. Areas that appear dark indicate regions where the bacteria's growth was unhindered by the antibiotic concentration. Areas that appear light are similar to the initial snapshot (t=0) and indicate regions where the antibiotic concentration was high enough to inhibit growth.

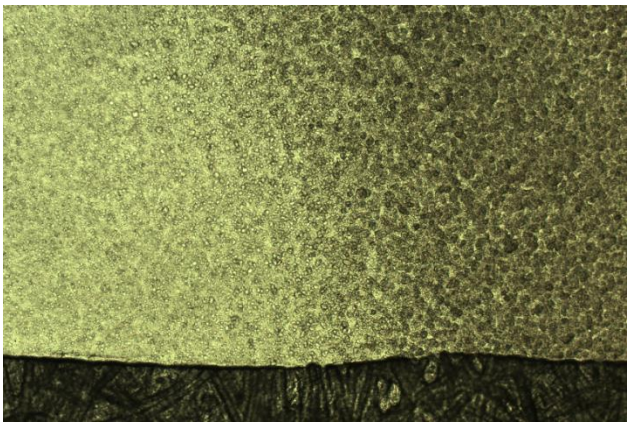

Figure 17: Snapshot image of the solid inoculated agar taken under light microscopy at 4x magnification after 8 hours of incubation.

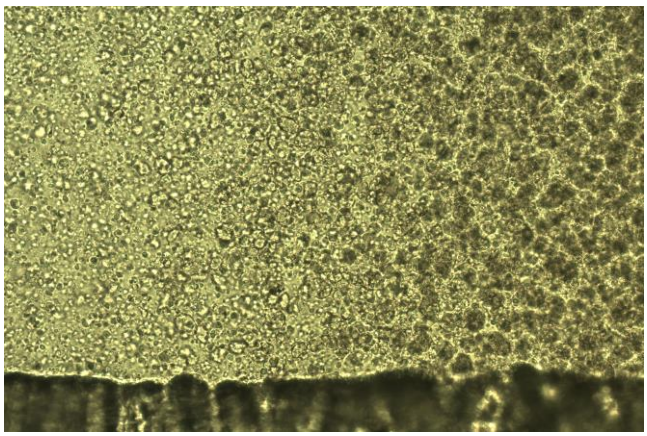

Figure 18: Snapshot image of the solid inoculated agar taken under light microscopy at 10x magnification after 8 hours of incubation.

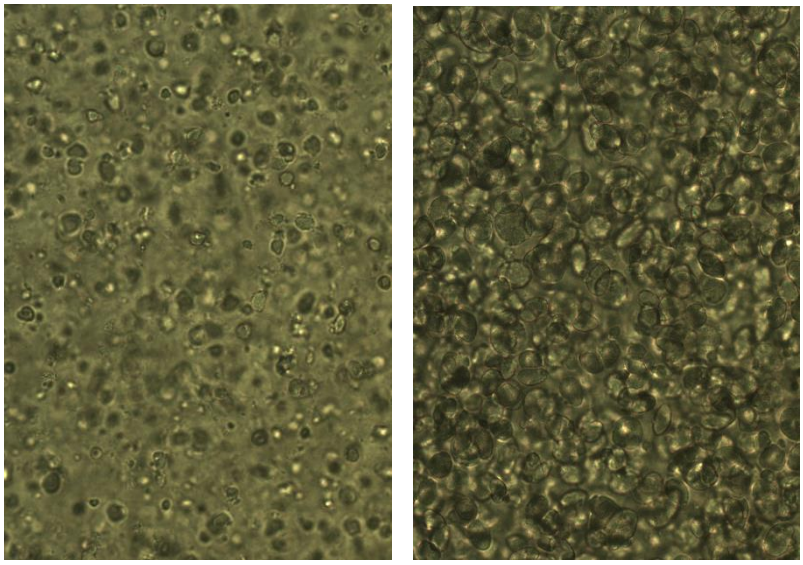

Figure 19: Two snapshot images of the solid inoculated agar taken under light microscopy at 20x magnification after 8 hours of incubation. Growth is pictured on the right and inhibition is pictured on the left.

Discussion

Discussion of the Preliminary Results

The final optimized design of the microfluidic device mold (Figure 11) was able to produce a relatively transparent and structurally functional device using PDMS (Figure 12). As is seen with Figures 15-19, the rapid antibiotic susceptibility test device prototype was able to produce a clear zone of inhibition that represented the minimal inhibitory concentration of an antibiotic against a population of bacteria. This suggests that our device has the potential to achieve the goal of rapidly analyzing the antibiotic susceptibility profile of any bacterial sample under a given antibiotic as the device has proved to be highly efficacious in achieving rapid AST.

The functioning device was imaged using standard light microscopy at 4x and 10x magnification. While this method provided satisfactory results, it required the inoculated agar to be incubated for a lengthy 8 hours before results could accurately be depicted. To decrease the amount of time required to establish antibiotic susceptibility profiles, a higher imaging magnification is recommended. Viewing the bacterial sample at the cell-level will enable observers to document frequencies of binary fission in as little as 20 minutes (the generation time of *E. coli*) per cell. The absence of growth in any allotted timespan indicates effective inhibition of a concentration of antibiotic. Light microscopy images were taken at 20x

magnification with similar results (Figure 19), but the resultant field-of-view was too small to image both the zone of inhibition and overgrowth boundaries. We conclude that light microscopy is not an efficacious imaging method when considering rapid AST systems. Thus, it is highly recommended that researchers utilize a low-cost lensless galvo speckle-scanning system, which was originally intended (Figure 20). While our device is capable of achieving low-cost antibiotic susceptibility testing, it is currently limited by the time required to incubate and image the growth of bacterial populations.

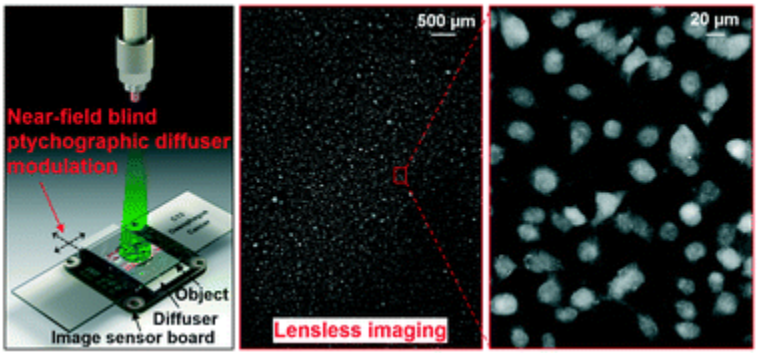

Figure 20: Wide-field, high-resolution lensless speckle-scanning system created using near-field blind ptychographic modulation [19]

Social, Economic, Political, and Cultural Concerns

The results of our project would ultimately reduce the economic toll on healthcare as a result of multidrug-resistant pathogens. Antibiotic resistance poses a huge threat to the economy as more and more people become resistant to doses and prescriptions that have otherwise efficiently treated the prognosis in the past. Specifically, if a patient is resisting usual antibiotic treatment and needs another prescription, their length of hospital stay will increase and the previous treatment will have been a wasted expense. Our project will work to determine the specific antibiotic and concentration that will treat the infection in as little time as possible, thus reducing the possibility of recurrent hospital visits due to inefficacious treatments. The speed of this device will allow a decrease in hospital time and cut out the incorrect prescription stage. This device will serve as an inexpensive alternative to costly antibiotic susceptibility testing assays and ultimately reduce the burden on both hospital staff and its patients through rapid and effective treatment protocols. It will also eliminate the need to prescribe expensive last-line antibiotics as a result of improper initial treatment.

Environmentally, the greatest cause of resistance comes from the return of antibiotics to nature. This occurs when antibiotics are discarded as waste or excreted as feces by way of sewage or landfill, which then leads to the growth of resistant bacteria. Our project seeks to decrease the amount of waste by making prescriptions more efficient and scarce. By reducing either the dosage or type of antibiotic being ingested, the effect of the environment can be slightly improved.

The project will have a high societal impact on how ordinary individuals are diagnosed and treated. The ultimate aim of this project is to reduce the morbidity of bacterial infections and thus reduce the burden and potential of disease in society. In theory, instead of attending a minute clinic for an infection and receiving a basic prescription for the presumed prognosis, a more specific dosage and antibiotic can be identified. For the ordinary person, this can decrease the time spent trying to get over an infection that is impacting their everyday life. This, of course, is the end goal, and will more readily be used to identify the effectiveness of an antibiotic for more exotic infections vs. the everyday.

Traditional antibiotic susceptibility testing platforms standardized, patented, and mass-produced. As a result, simple tests such as the broth microdilution and ETest may cost upwards of $5 per test (well/strip) for a single antibiotic. Novel rapid AST platforms are capable of reducing the time required to prescribe effective treatment but often require specialized machinery and equipment that can well exceed several thousand USD. Our device would compete in the global market for antibiotic susceptibility tests and imaging devices at a fraction of the cost of traditional platforms and systems. As a result, it would be suited for all economies regardless of affluence. Religions or cultures that are not accepted of blood sampling would likely reject this device. However, our device may be extrapolated to utilize other biological samples, such as mucous or feces.

Our device does not collect any confidential information or data other than the antibiotic susceptibility profiles of a patient. Collected information is limited to data protected under HIPAA regulations, often used to label and track clinical samples. The testing protocol is simple and minimally invasive. All that is required to conduct rapid AST is a clinical sample, taken from either the blood or mucous. We aim for beneficence and nonmaleficence by prescribing the most efficacious treatment possible and preventing the need for recurrent hospitalizations due to improper treatment.

Our project aims to develop a new antibiotic susceptibility testing platform to significantly decrease the waiting time for results. Current methods on the market can take anywhere from 12-36 hours to yield results, whereas our product delivers in 1-2 hours. The

the survival rate of an individual patient decreases by 7.6% with every hour that passes so it is critical that the testing is completed as quickly as possible. This will have a tremendous impact on the health and personal safety of people worldwide as antibiotic resistance takes hundreds of thousands of lives every year.

Our design is easily manufactured at a low cost. The mold is 3D printed from PLA fibers, which are very affordable. The device itself is made from PDMS, a widely available silicon with a high curing rate. This antibiotic susceptibility testing system also incorporates a low-cost lensless galvo speckle-scanning device. This imaging system comes at a fraction of the cost of traditional light microscopes and provides greater resolution and magnification. The design is straightforward and could be easily reproduced for clinical use.

The mold of the device takes sustainability efforts into account as it is printed using organic, renewable material and it can be reused to fabricate several PDMS devices. PLA can decompose and degrades into non-harmful byproducts. However, the PDMS device is single-use and must be disposed of after testing. Thus, it is recommended that the mold be manufactured out of metal and the device be cast out of reusable acrylic or glass.

Future Implications

It is recommended that the final optimized mold be manufactured out of metal to further prevent the issue of air-bubble formation in the cured PDMS device. The utilization of 3D or resin printers often results in poor resolution limited by filament size. A metal mold may be smoothed out to prevent any surface discrepancies/irregularities that could house microscopic air pockets. A stronger vacuum chamber may also be used to remove small air pockets from the uncured PDMS. Additionally, the current utilization of PDMS results in a flexible but relatively soft and delicate device structure. As future development of the device should focus on minimizing the prototype to true microfluidic applications, we recommend that an acrylic device be used in place of PDMS to improve both transparency and structural integrity.

The proof-of-concept for the microfluidic device was limited to a population of *E. coli* tested against an ampicillin solution under laboratory conditions. To help achieve the goal of rapid AST, we recommend that the bacterial population be a direct clinical blood sample treated with anticoagulants. This sample may be diluted with brain and heart infusion to support fastidious bacterial growth, and then filtered using a 0.45μm membrane to isolate bacterial populations. From there, the filtered bacterial samples may be used to inoculate brain-and-heart agar, which may then be poured into the device for rapid AST applications. This would minimize the time required to establish antibiotic susceptibility profiles by eliminating the need to

centrifuge whole blood, isolate bacterial populations, proliferate (incubate) the bacteria, and then test their antibiotic susceptibility profiles.

Finally, it is recommended that the antibiotic concentration gradient be established using a non-porous strip, or on top of a non-porous barrier. The current porous membrane wicks moisture away from the agar before the concentration gradient can fully establish. This would ultimately alter the diffusion approximations and result in inaccurate calculations.

References

[1]E. Tacconelli, E. Carrara, A. Savoldi, S. Harbarth, M. Mendelson, D. L. Monnet, C. Pulcini, G. Kahlmeter, J. Kluytmans, and Y. Carmeli, "Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis," The Lancet Infectious Diseases 18, 318-327 (2018).

[2]Sterling, S. A., Miller, W. R., Pryor, J., Puskarich, M. A., & Jones, A. E. (2015). The Impact of Timing of Antibiotics on Outcomes in Severe Sepsis and Septic Shock: A Systematic Review and Meta-Analysis. *Critical care medicine*, *43*(9), 1907–1915.

[3]M. E. A. de Kraker, A. J. Stewardson, and S. Harbarth, "Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050?," PLOS Medicine 13, e1002184 (2016).

[4]Avesar, J., Rosenfeld, D., Truman-Rosentsvit, M., Ben-Arye, T., Geffen, Y., Bercovici, M., & Levenberg, S.(2017). Rapid phenotypic antimicrobial susceptibility testing using nanoliter arrays. *Proceedings of the National Academy of Sciences*, *114*(29), E5787-E5795.

[5]Puttaswamy, S., Gupta, S. K., Regunath, H., Smith, L. P., & Sengupta, S. (2018). A comprehensive review of the present and future antibiotic susceptibility Testing (AST) systems. *Arch Clin Microbiol*, *9*.

[6]Davies, J., & Davies, D. (2010). Origins and evolution of antibiotic resistance. Microbiology and molecular biology reviews : MMBR, 74(3), 417–433.

[7]Baltekin, Ö., Boucharin, A., Tano, E., Andersson, D. I., & Elf, J. (2017). Antibiotic susceptibility testing in less than 30 min using direct single-cell imaging. *Proceedings of the National Academy of Sciences*, *114*(34), 9170-9175.

[8]Toprak, E., Veres, A., Michel, J. B., Chait, R., Hartl, D. L., & Kishony, R. (2012). Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nature genetics*, *44*(1), 101.

[9] C. M. Buchanan, R. L. Wood, T. R. Hoj, M. Alizadeh, C. G. Bledsoe, M. E. Wood, D. S. Mcclellan, R. Blanco, C. L. Hickey, T. V. Ravsten, G. A. Husseini, R. A. Robison, and W. G. Pitt, “Rapid separation of very low concentrations of bacteria from blood,” *Journal of Microbiological Methods*, vol. 139, pp. 48–53, 2017.

[10] Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D., & Buret, A. (1999). The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *Journal of clinical microbiology*, *37*(6), 1771-1776.

[11] Choi, J., Jung, Y. G., Kim, J., Kim, S., Jung, Y., Na, H., & Kwon, S. (2013). Rapid antibiotic susceptibility testing by tracking single cell growth in a microfluidic agarose channel system. *Lab on a Chip*, *13*(2), 280-287.

[12] Maxson, T., Blancett, C. D., Graham, A. S., Stefan, C. P., & Minogue, T. D. (2018). Rapid antibiotic susceptibility testing from blood culture bottles with species agnostic real-time polymerase chain reaction. *PloS one*, *13*(12), e0209042.

[13] Mohan, R., Mukherjee, A., Sevgen, S. E., Sanpitakseree, C., Lee, J., Schroeder, C. M., & Kenis, P. J. (2013). A multiplexed microfluidic platform for rapid antibiotic susceptibility testing. *Biosensors and Bioelectronics*, *49*, 118-125.

[14] S. Puttaswamy, B. D. Lee, and S. Sengupta, “Novel Electrical Method for Early Detection of Viable Bacteria in Blood Cultures,” Journal of Clinical Microbiology, vol. 49, no. 6, pp. 2286–2289, Jun. 2011.

[15] Sauget, M., Bertrand, X., & Hocquet, D. (2018). Rapid antibiotic susceptibility testing on blood cultures using MALDI-TOF MS. *PloS one*, *13*(10), e0205603.

[16] Tang, Y., Zhen, L., Liu, J., & Wu, J. (2013). Rapid antibiotic susceptibility testing in a microfluidic pH sensor. *Analytical chemistry*, *85*(5), 2787-2794.

[17] W. G. Pitt, M. Alizadeh, G. A. Husseini, D. S. Mcclellan, C. M. Buchanan, C. G. Bledsoe, R. A. Robison, R. Blanco, B. L. Roeder, M. Melville, and A. K. Hunter, “Rapid separation of bacteria from blood-review and outlook,” *Biotechnology Progress*, vol. 32, no. 4, pp. 823–839, Mar. 2016.

[18] Wimmer, J. L., Long, S. W., Cernoch, P., Land, G. A., Davis, J. R., Musser, J. M., & Olsen, R. J. (2012). Strategy for rapid identification and antibiotic susceptibility testing of gram-negative bacteria directly recovered from positive blood cultures using the Bruker MALDI Biotyper and the BD Phoenix system. *Journal of clinical microbiology*, *50*(7), 2452-2454.

[19] S. Jiang, J. Zhu, P. Song, C. Guo, Z. Bian, R. Wang, Y. Huang, S. Wang, H. Zhang, and G. Zheng, “Wide-field, high-resolution lensless on-chip microscopy via near-field blind ptychographic modulation,” Lab on a Chip, vol. 20, no. 6, pp. 1058–1065, 2020.